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Characterization of a novel endolysin LysSA11 and its utility as a potent biocontrol agent against *Staphylococcus aureus* on food and utensils

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ABSTRACT

Here we show that the LysSA11 endolysin, derived from the virulent *Staphylococcus aureus* phage SA11, has lytic activity against staphylococcal strains. Bioinformatics analysis revealed an enzymatically active CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain at the N-terminus of LysSA11 that showed amidase activity. A novel cell wall binding domain (CBD) in the C-terminus could bind to a broad spectrum of staphylococcal cells. The bactericidal activity of LysSA11 was determined in food and utensils artificially contaminated with methicillin-resistant *S. aureus* (MRSA). The amounts of MRSA bacteria in milk and on ham were significantly reduced by 1.44-log CFU/mL and 3.12-log CFU/cm^3, respectively, within 15 min at refrigeration temperature (4 °C) and by 2.02-log CFU/mL and 3.37-log CFU/cm^2, respectively, within 15 min at room temperature (25 °C). Moreover, a polypropylene plastic cutting board and a stainless steel knife artificially contaminated with approximately 4-log CFU/cm^2 of MRSA also showed complete bacterial elimination after a 30-min treatment with 1.35 μM of LysSA11. The data presented here strongly suggest that the novel CBD-containing staphylococcal endolysin LysSA11 can be used both as a food antimicrobial and as a practical sanitizer for utensils.

1. Introduction

Staphylococcus aureus is a highly virulent pathogen that produces heat-stable enterotoxins responsible for staphylococcal food poisoning (Dinges et al., 2000; Le Loir et al., 2003). A number of diseases caused by consumption of contaminated milk and milk products have been reported since the early 20th century, including staphylococcal toxemia (Crabtree and Litterer, 1934; Denison, 1936; Hennekinne et al., 2012). *S. aureus*-mediated food poisoning usually results from consumption of milk and dairy products produced by a cow suffering from mastitis caused by *S. aureus* (Kümmel et al., 2016). This type of food poisoning is one of the major food safety concerns worldwide (Gruet et al., 2001). *S. aureus* is also a frequent cause of food poisoning from ham products (Richards et al., 1993). For example, staphylococcal food poisoning outbreak occurred at an International equine sports event in Luxembourg (Mossong et al., 2015) and microbiological investigations revealed that ham was one of the main vehicle for the staphylococcal intoxication. In addition, various foods including milk, meat, and salad were reported to be the cause of staphylococcal food poisoning (Hennekinne et al., 2012). Moreover, *S. aureus* foodborne disease outbreaks are facilitated by cross-contamination during food preparation and processing in the kitchen. From this perspective, insufficient cleaning of cooking utensils and contamination of food storage environments were the most common errors reported (Bennett et al., 2013; Kadariya et al., 2014). The emergence of antibiotic-resistant *S. aureus* strains, including methicillin-resistant

S. aureus (MRSA), has further complicated the treatment of *S. aureus* infections (Seaton et al., 2014).

Bacteriophage-encoded peptidoglycan hydrolases called endolysins directly target bonds in the bacterial cell wall peptidoglycan. These enzymes are synthesized at the end of the phage life cycle to lyse the host cell and allow the release of newly produced virions into the environment (Bai et al., 2016; Gu et al., 2011b; Schmelcher et al., 2015). While the use of bacteriophage particles as antimicrobials is associated with the risk of harmful gene transduction or development of phage-resistant pathogens, treatment with phage-derived endolysins does not have such concerns. Therefore, endolysins are promising biocontrol agents that could be applied in the field of food safety (Obeso et al., 2008; Oliveira et al., 2012).

Despite these benefits, only a few studies have assessed the potential of endolysins as food additives (Garcia et al., 2010; Obeso et al., 2008). One report found that treatment with 160 U/mL LysH5 endolysin (88 μg/mL) for 1 h at 37 °C resulted in an approximately 1 log CFU/mL reduction of *S. aureus* (Obeso et al., 2008). Studies in other foods have not yet been performed, even though *S. aureus* is regarded as a frequent cause of food poisoning from ham products (Richards et al., 1993). Moreover, no report has yet investigated the potential of endolysins as disinfectants that could be used to treat cooking utensils. In this study, we purified and characterized the staphylococcal endolysin LysSA11 from the virulent phage SA11 (Kim and Myung, 2012) to use it as alternative antimicrobials or sanitizers in the practical application. This enzyme is comprised of a CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain as well as a novel cell wall binding domain. We demonstrate that LysSA11 shows strong anti-staphylococcal activity in food (pasteurized milk and ham) and on utensils (polypropylene plastic cutting board and stainless steel knife). This is the first study to examine the possibility of using novel CBD-containing endolysin LysSA11 as both a food antimicrobial agent and sanitizer.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

The bacterial strains used in this study are summarized in Table 1. All strains were grown in tryptic soy broth (TSB) medium (Difco, Detroit, MI) at 37 °C with aeration. Baird-Parker agar plates with egg yolk tellurite (BPA; Difco) were used for staphylococcus colony counting. *Escherichia coli* DH5α and BL21 (DE3) strains, used for cloning of the endolysin gene and expression of the endolysin gene, respectively, were grown in Luria-Bertani (LB) broth (Difco) at 37 °C with aeration.

2.2. Bioinformatics analysis

From the complete genome sequence of the *S. aureus* phage SA11 (NCBI accession number NC_019511.1), a gene (*SA11_gp172*) encoding the LysSA11 endolysin was identified. The domain architecture of LysSA11 was analyzed using InterProScan5 (Ziedaite et al., 2005). The amino acid sequences of several staphylococcal endolysins harboring the CHAP domain were aligned using BLAST (Altschul et al., 1997) or Clustal X2 (Larkin et al., 2007).

2.3. Cloning and purification of LysSA11 and LysSA11-CBD

The endolysin gene (*SA11_gp172*) was PCR amplified using the following primers: lysSA11_forward (5′-AAGGAGTGAAAACA-TATGAAAGCATCGATG-3′) and lysSA11_reverse (5′-GGAAAATTCCCTAGTCTCGAGTTTCCAGTTAATACG-3′) (restriction sites are underlined). The resulting PCR product was subcloned into pET29b (Novagen, Madison, WI), which harbors a C-terminal hexahistidine (6 × His)-tag sequence. For LysSA11 endolysin expression, *E. coli* BL21 (DE3) cells were transformed with the resulting plasmid. Protein expression was induced by the addition of 0.5 mM isopropyl-β-thiogalactopyranoside (final concentration) upon reaching an OD$_{600}$ of 0.8, after which cells were further incubated at 18 °C for 22 h. Cells were suspended in lysis buffer (20 mM sodium phosphate, 200 mM NaCl, pH 8.0) and lysed by sonication (Branson Ultrasonics, Danbury, CT). The clarified supernatant containing soluble proteins was obtained by centrifugation at 15,000 × g for 30 min, followed by filtration (0.22-μm pore size; Millipore). Recombinant protein was purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen Gmbh, Germany) according to the manufacturer's instructions. The identity and purity of the protein were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Dunne et al., 2014). Purified protein was exchanged into storage buffer (20 mM sodium phosphate, 200 mM NaCl, 30% glycerol, pH 8.0) using a PD Miditrap G-25 (GE Healthcare, Amersham, Bucks, UK) and stored at −80 °C until use.

The region encoding the predicted cell wall binding domain (LysSA11-CBD) was PCR amplified using the following primers: LysSA11-CBD-forward (5′-TCAAAGACGAGGGATCCACAACTTGGA-3′) and LysSA11-CBD-reverse (5′-AAAATTCCCTCTCGAGTTATTTC-CAGT-3′) (restriction sites are underlined). The resulting PCR product was subcloned into pET28a-EGFP, which encodes the 6 × His tag and enhanced green fluorescent protein (EGFP), thereby constructing an EGFP-LysSA11-CBD fusion protein. Recombinant EGFP-LysSA11-CBD was expressed and purified as described above.

2.4. Characterization of the LysSA11 endolysin

The lytic activity of LysSA11 was assessed by the turbidity reduction assay (Son et al., 2012). Briefly, exponentially growing cells (*S. aureus* ATCC 13301) were harvested and suspended in reaction buffer (50 mM sodium phosphate, 200 mM NaCl, pH 8.0) to an OD$_{600}$ of approximately 1.0 (Gaeng et al., 2000). After the addition of purified endolysin (45 nM—450 nM), the OD$_{600}$ values were periodically monitored (0, 10, 20, 30, 40, 50, and 60 min). For Gram-negative bacteria, exponentially growing cells were pretreated with a buffer containing 50 mM sodium phosphate, 200 mM NaCl (pH 8.0), and 100 mM EDTA for 5 min at room temperature (RT). The cells were washed three times with reaction buffer to remove residual EDTA (Leive, 1968) prior to endolysin addition.

To test the susceptibility of cells to LysSA11-mediated killing in various pHs, 225 nM of LysSA11 was added to *S. aureus* ATCC 13301 cell suspensions in the following buffers: 0.1% trifluoroacetic acid (pH 2.0), 50 mM sodium acetate (pH 4.0), 50 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.0), 50 mM Bis-Tris (pH 7.0), 50 mM Tris-HCl (pH 8.0), 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (pH 9.0), and 50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (pH 10.0) (Park et al., 2012). To assess the effect of temperature on LysSA11 enzymatic activity, endolysins incubated at different temperatures (4—65 °C) were used in the turbidity reduction assay. The influence of NaCl on the lysis activity was evaluated by using different NaCl concentrations (0—300 mM) (Son et al., 2012).

The effects of metal ions on lysis activity were determined as previously reported (Schmelcher et al., 2012). To chelate metal ions attached to the endolysin, thereby inhibiting its catalytic function, EDTA (5.0 mM; final concentration) was added to the endolysin (225 nM) and incubated at 37 °C for 1 h. The EDTA was then removed by exchanging the endolysin into 50 mM phosphate buffer (pH 8.0) using a PD trap G-25 column. The EDTA-treated enzyme was added to cell suspensions with metal ions (CaCl$_2$,

Table 1
Antimicrobial spectra of the SA11 phage and LysSA11 endolysin, and the cell wall domain binding spectrum of LysSA11-CBD.

Bacterial strain	Host range of SA11	Lysis zone formation by LysSA11	Binding activity of LysSA11-CBD	Reference or source[b]
Staphylococcus strains				
Staphylococcus aureus ATCC 13301	+	+	+	ATCC
MRSA CCARM 3089	+	+	+	CCARM
MRSA CCARM 3090	+	+	+	CCARM
MRSA CCARM 3793	+	+	+	CCARM
Staphylococcus aureus KCTC 1916	+	+	+	KCTC
Staphylococcus aureus ATCC 6538	+	+	+	ATCC
Staphylococcus aureus ATCC 23235	+	+	+	ATCC
Staphylococcus aureus ATCC 29213	+	+	+	ATCC
Staphylococcus aureus ATCC 25923	+	+	+	ATCC
Staphylococcus aureus Newman	+	+	+	(Baba et al., 2008)
Staphylococcus aureus ATCC 33586	+	+	+	ATCC
Staphylococcus aureus RN4220	+	+	+	(Park et al., 2010)
Staphylococcus aureus ATCC 33593	+	+	+	ATCC
Staphylococcus aureus ATCC 12600	+	+	+	ATCC
Staphylococcus aureus food isolate (from vegetable)	+	+	+	this study
Staphylococcus aureus food isolate (from beef)	+	+	+	this study
Other Gram positive bacteria				
Enterococcus faecalis ATCC 29212	–	–	–	ATCC
Bacillus cereus ATCC 14579	–	–	–	ATCC
Bacillus subtilis ATCC 23857	–	–	–	ATCC
Listeria monocytogenes ATCC 19114	–	–	–	ATCC
Gram negative bacteria				
Salmonella enterica serovar Typhimurium SL1344	–	–[a]	–[a]	ATCC
Escherichia coli MG1655 ATCC 47076	–	–[a]	–[a]	ATCC
Escherichia coli O157:H7 ATCC 35150	–	–[a]	–[a]	ATCC
Cronobacter sakazakii ATCC 29544	–	–[a]	–[a]	ATCC
Pseudomonas aeruginosa ATCC 27853	–	–[a]	–[a]	ATCC

[a] Gram-negative bacteria were treated with EDTA.
[b] ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Culture; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

MgCl$_2$, MnCl$_2$, CuCl$_2$, or ZnCl$_2$; 1.0 mM final concentration), and the lysis activity was assayed in reaction buffer.

2.5. Plate lysis assay

Plate lysis assays were performed as previously described (Becker et al., 2009). In brief, 10 μL of diluted endolysin in reaction buffer (0.5 μg/μL; final concentration) was spotted onto a freshly prepared bacterial lawn at mid-log phase on Tryptic Soy Agar (TSA) plates. Plates were air-dried in a laminar flow hood for 15 min and incubated overnight at 37 °C. Cleared spots were scored within 20 h of plating. Lysis, as indicated by a clear bacterial zone, was considered a positive result.

2.6. Amidase assay

N-acetylmuramoyl-L-alanine amidase activity was measured by quantification of endolysin-mediated release of acetaldehyde from peptidoglycan. S. aureus ATCC 13301 peptidoglycan was prepared as previously described (Fein and Rogers, 1976; Kuroda and Sekiguchi, 1990). Bacterial cells were disrupted by sonication and centrifuged at low speed (1400 × g, 10 min) to remove unbroken cells. The resultant supernatant was centrifuged again at high speed (27,000 × g, 5 min), and the crude cell wall pellet was boiled for 10 min in 4% SDS solution (wt/vol). After three washes with distilled water, the peptidoglycan fraction was resuspended in endolysin reaction buffer. Peptidoglycan solution and 225 nM of LysSA11 solution were used as negative controls; reaction buffer alone was used as the reference. For the experimental group, 200 μL of peptidoglycan solution (1 mg/mL) containing 225 nM of LysSA11 was prepared. All reactions were incubated at RT for 1 h, after which 1.0 M NaOH was added to each reaction to stop the reaction. After 30 min incubation at 38 °C, 500 μL of 0.5 M H$_2$SO$_4$ and 5 mL of

concentrated sulfuric acid were added sequentially to each reaction. The stoppered reaction tubes were placed in boiling water for 5 min. After cooling on ice, 4% CuSO$_4$·5H$_2$O and 1.5% ρ-hydroxydiphenyl solutions were added, and the mixtures were incubated for 30 min at 30 °C. Finally, the activity of N-acetylmuramoyl-L-alanine amidase was calorimetrically assessed by measuring the OD$_{560\ nm}$ of the supernatants (Hadzija, 1974; Hazenberg and de Visser, 1992).

2.7. EGFP fusion protein binding assay

Cells at early exponential growth phase (1 mL) were harvested and resuspended in 100 μL of phosphate-buffered saline (PBS). After incubation with 0.8 μM of EGFP-LysSA11-CBD for 5 min at RT, the cells were collected by centrifugation at 16,000 × g for 1 min, washed, and resuspended in PBS. EGFP alone was used as a negative control to test whether the EGFP tag had any binding affinity to the bacterial cell surface. A super-resolution confocal microscope (Leica, SP8 X STED, Germany) was used to detect the fluorescence from the treated cells to determine the binding specificity of LysSA11-CBD (Gu et al., 2011a).

2.8. Preparation of food samples and antimicrobial activity assay of LysSA11

The lytic activity of LysSA11 against MRSA CCARM 3089 strain was tested in commercial pasteurized milk and ham those were purchased from a local market (Seoul, Korea). A sample (4 mL) of fresh milk pasteurized at 63 °C for 30 min was inoculated with 1 mL of MRSA CCARM 3089 cells (2 × 10^5 CFU/mL) at exponential growth phase. Before the addition of LysSA11 at 0, 1.125, 2.25, 3.375, 4.5, and 9 μM, the milk samples were pre-incubated at 4 °C (refrigeration temperature) or 25 °C (RT) for 1 h to allow the bacteria to

adapt to each condition. Each milk sample was then incubated at 4 °C or 25 °C for an additional hour. Viable bacterial cells (CFU/mL) were counted every 15 min after LysSA11 addition by plating each sample on a BPA plate, a selective medium for *Staphylococcus* spp. identification. The absence of *S. aureus* in non-inoculated milk was verified by direct plating onto a BPA plate.

Ham samples were aseptically cut into cubes approximately 0.5 cm^3 in volume. MRSA CCARM 3089 cells (approximately 2×10^5 CFU/mL) were inoculated onto the surface of each ham sample by pipetting and were pre-incubated at 4 °C or 25 °C for 1 h to allow the bacteria to adapt to each condition. Subsequently, each ham sample was inoculated with 0–9 μM of LysSA11 and further incubated at 4 °C or 25 °C for an additional hour. Viable bacterial cells (CFU/cm^3) on the ham were counted every 15 min after LysSA11 addition. At each time point, *S. aureus* cells were detached from the ham surface by agitating the ham in PBST (PBS containing 0.05% Tween-20) for 2 min with a benchtop vortex mixer at maximum speed. Cell suspensions were serially diluted and plated onto BPA plates. The absence of *S. aureus* from a non-inoculated ham sample was verified by direct plating and used as a negative control.

2.9. Preparation of cooking utensils and antimicrobial activity test of LysSA11

A polypropylene cutting board and a stainless steel knife were sterilized with 70% alcohol (vol/vol %). Exponentially growing MRSA CCARM 3089 bacterial cells were harvested and resuspended in PBS to a final concentration of approximately 10^5 CFU/mL. Prepared bacterial cells (1 mL) were pipetted onto the surfaces (4 cm^2 squares) of the cooking utensils and dried for 1 h aseptically. Subsequently, each region was treated with LysSA11 (0–1.35 μM) and left for 30 min at RT. For the negative control, PBS was used instead of LysSA11 solution. To recover the bacteria, swabs pre-moistened with PBST were swiped methodically 10 times in each horizontal, vertical, and diagonal direction. After sampling, swabs were placed
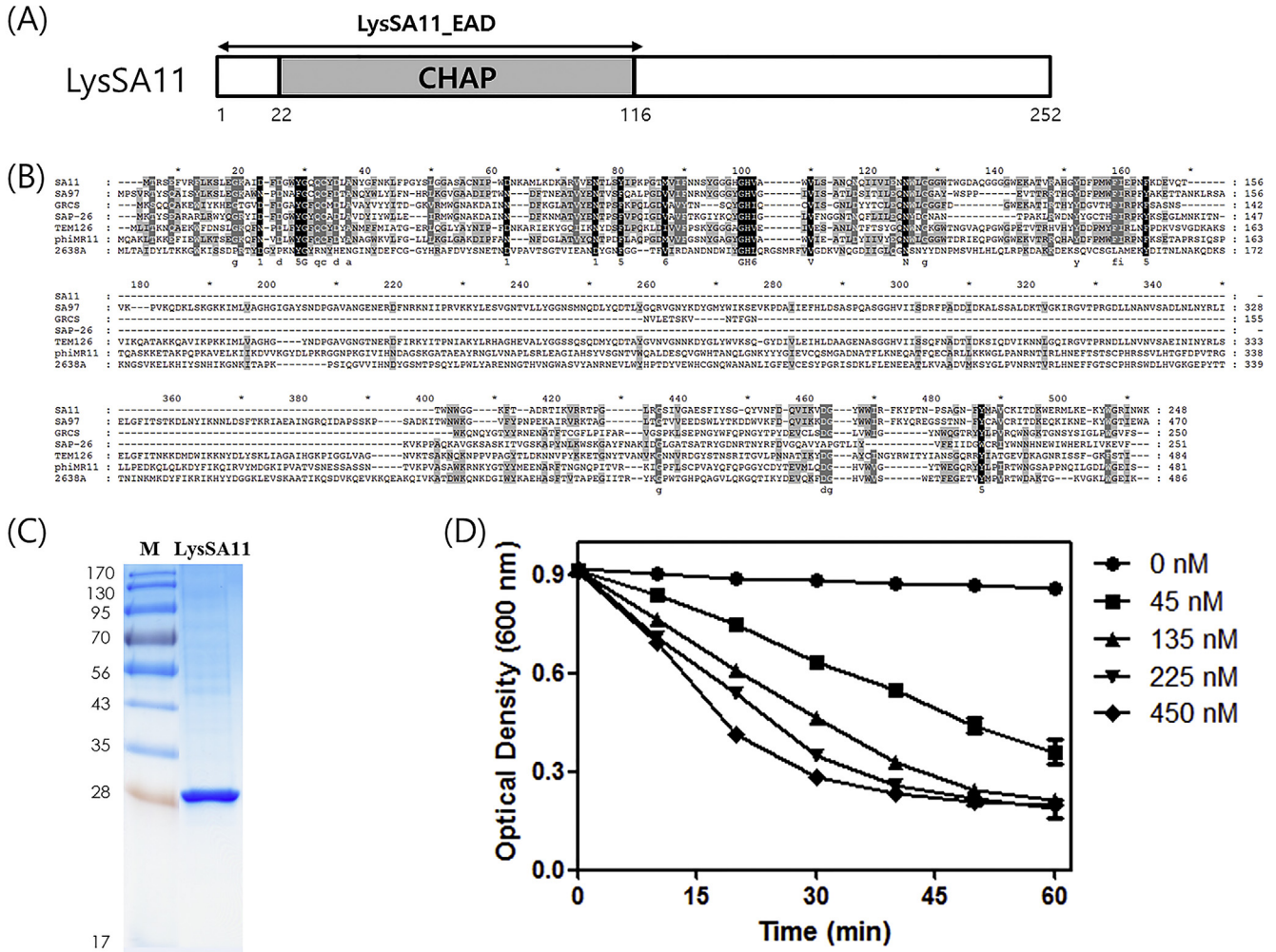
Fig. 1. Modular structure and lytic activities of the LysSA11 endolysin from *S. aureus* phage SA11. (A) Schematic representation of LysSA11. The conserved N-terminal cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain is shown. (B) Sequence alignment of various staphylococcal phage endolysins. SA11, SA11 phage endolysin containing a CHAP domain; SA97, SA97 phage endolysin containing CHAP and amidase_3 domains; GRCS, GRCS phage endolysin containing CHAP and SH3_5 domains; SAP-26, SAP-26 phage endolysin containing a CHAP domain; TEM126, TEM126 phage endolysin containing CHAP, amidase_3, and SH3_5 domains; phiMR11, phiMR11 phage endolysin containing CHAP, amidase_2, and SH3_5 domains; and 2638A, 2638A phage endolysin containing peptidase_M23, amidase_2, and SH3_5 domains. Conserved and identical residues are shaded in gray (dark gray, >70% conserved; light gray, >40% conserved) and black, respectively. (C) SDS-PAGE analysis of purified LysSA11. M, standard molecular weight marker; LysSA11, purified LysSA11 fraction. (D) Lysis of *S. aureus* ATCC 13301 cells treated externally with various concentrations of recombinant LysSA11. Optical density was measured periodically after LysSA11 treatment.

into tubes containing PBST and then agitated with a benchtop vortex mixer at maximum speed for 2 min. The suspensions were serially diluted, plated onto BPA plates, and incubated at 37 °C for 24 h.

2.10. Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 5.01). and InStat v.3.1 programs (GraphPad, San Diego, CA, USA). The one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test (95% confidence interval) were done. The data are presented as means with standard deviations. A P-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Bioinformatics analysis of LysSA11

Phage SA11 endolysin LysSA11 (Kim and Myung, 2012), a protein composed of 252 amino acids (28.82 kDa), was subjected to domain analysis using Pfam 28.0. The analysis revealed that the endolysin contains a cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain (PF05257; E-value, 2.2e-11) at its N-terminus (Fig. 1A). This is the enzymatic domain that cleaves the amide bond between N-acetylmuramic acid and L-alanine in the bacterial cell wall (Szweda et al., 2012). BLAST analysis revealed that LysSA11 shows the closest homology to an amidase from S. aureus phage vB_SauM_Romulus (YP_007677654.1, 98% identity) (Vandersteegen et al., 2013) at the amino acid sequence level. No other homologous proteins with an identity higher than 60% were found. Comparison of the LysSA11 amino acid sequence to those of previously reported endolysins revealed that LysSA11 showed low identity with other endolysins. These endolysins could be classified into separate groups based on domain composition (Chang and Ryu, 2016): endolysins from SA97 (47.0% identity; KJ716334.1; containing CHAP and amidase_3 domains), GRCS (21.5%; KJ210330.1; containing CHAP and SH3_5 domains), SAP-26 (20.6%; NC_014460.1; containing a CHAP domain), TEM126 (26.0%; HQ127381.1; containing CHAP, amidase_3, and SH3_5 domains), phiMR11 (31.9%; NC_010147.1; containing CHAP, amidase_2, and SH3_5 domains), and 2638A (14.6%; NC_007051.1; containing peptidase_M23, amidase_2, and SH3_5 domains) (Fig. 1B). These results suggest that LysSA11 is a novel endolysin that can be classified as a new type of staphylococcal endolysin.

The soluble fraction of LysSA11 expressed in E. coli was purified by nickel affinity chromatography via its C-terminal 6 × His tag (Fig. 1C). To confirm the predicted amidase activity of LysSA11, the amount of free muramic acid generated after endolysin treatment of peptidoglycan was measured calorimetrically (Hadzija, 1974). Lactic acid derived from free muramic acid by 0.5 M sulfuric acid treatment was decarboxylated using concentrated sulfuric acid. Subsequently, the resultant acetaldehydes were treated with ρ-hydroxydiphenyl, resulting in a color change of the solution to blue (Park et al., 2012). As shown in Fig. 2, the amount of free muramic acid released from S. aureus ATCC 13301 peptidoglycan was increased 5.8-fold by 1 h of LysSA11 treatment compared to the non-treated or substrate-free control groups. These results suggest that LysSA11 has amidase activity and is capable of cleaving the lactate link between N-acetylmuramic acid and the L-alanine of the peptide side chain.

Although most of the previously reported staphylococcal endolysins were shown to contain an SH3_5 domain as the CBD (Oliveira et al., 2013), neither InterProScan 5 nor Pfam 28.0 analysis of LysSA11 revealed a CBD homolog from the database. This finding suggests that LysSA11 has a novel CBD in its C-terminal region that
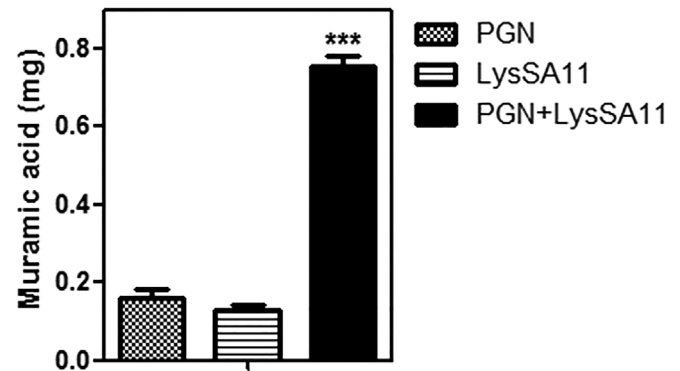
Fig. 2. N-acetylmuramoyl-L-alanine amidase activity of LysSA11. Peptidoglycan (PGN) extracted from S. aureus ATCC 13301 was incubated with or without 225 nM of purified LysSA11. The level of released muramic acid was measured calorimetrically after 1 h of incubation with the endolysin (details in Materials and Methods). PGN, peptidoglycan only; LysSA11, treatment with 225 nM LysSA11 without peptidoglycan; PGN + LysSA11, treatment with 225 nM LysSA11 and peptidoglycan. Each column represents the mean and standard deviation of triplicate assays, and the asterisks indicate significant differences (***, $P < 0.0001$).

is large enough to function as a CBD.

### 3.2. Binding activity of the C-terminal domain of LysSA11 as a CBD

To identify the specific bacterial binding activity of the putative CBD of LysSA11 (LysSA11-CBD), an EGFP fusion protein containing the predicted CBD region (amino acid residues 166–252) was cloned into pET28a and expressed in E. coli (Fig. 3A). Purified EGFP or EGFP-LysSA11-CBD was added to the bacterial cells, and protein binding was observed by fluorescence microscopy. While EGFP alone did not bind to S. aureus, the fusion protein bound specifically to S. aureus ATCC 13301 cells, causing fluorescence emission from the cell surface, as shown in Fig. 3B. LysSA11-CBD bound to all staphylococcal strains tested, including MRSA (Table 1). However, no fusion protein binding was observed to any other bacteria tested under the same conditions (Table 1). These results indicate that the LysSA11-CBD specifically targets staphylococcal cells.

### 3.3. Biochemical characterization of LysSA11

The LysSA11 endolysin showed lytic activity against exponentially growing S. aureus ATCC 13301 cells in a concentration-dependent manner (Fig. 1D). The highest dose (450 nM of endolysin) yielded a 50% reduction in optical density in less than 20 min and a 70% reduction within 30 min.

LysSA11 showed more than 60% residual lytic activity after incubation at temperatures ranging from 25 to 45 °C, while the activity was less than half of that at 4 °C or 65 °C (Fig. 4A). The endolysin retained more than 50% of its activity at pHs from 6.0 to 8.0, but its activity was markedly reduced at acidic pH (below 4.0) (Fig. 4B). Because the activities of several staphylococcal endolysins were reported to be enhanced by the addition of NaCl (Becker et al., 2008; Garcia et al., 2010), we investigated the activity of LysSA11 in the presence of NaCl at concentrations ranging from 0 to 500 mM. Similar to other staphylococcal endolysins, LysSA11 showed increased activity at up to 200 mM of NaCl (Fig. 4C). Based on the above observations, subsequent antimicrobial assays were performed at pH 8.0 in phosphate buffer containing 200 mM of NaCl. The results suggest that LysSA11 is a good candidate for food antimicrobials, particularly in neutral/moderately acidic food or in food containing sodium salts.

To examine whether divalent ions affect LysSA11 activity, the
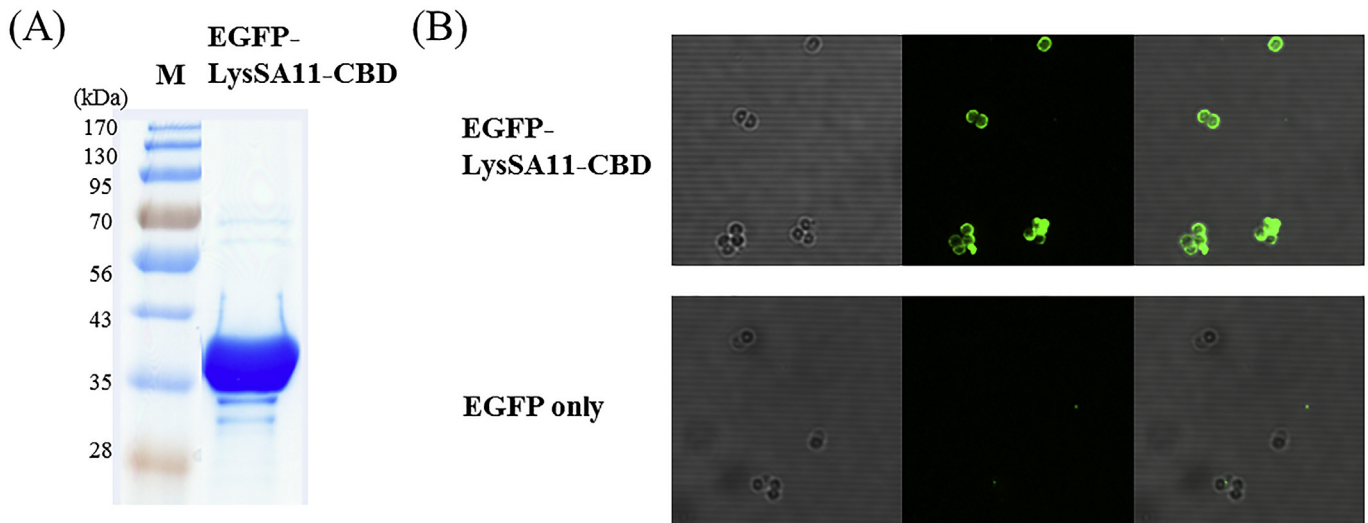
Fig. 3. Purification and binding activity of EGFP-LysSA11-CBD. (A) SDS-PAGE analysis of purified EGFP-LysSA11-CBD. M, standard molecular weight marker; EGFP-LysSA11-CBD, purified fraction of EGFP-LysSA11-CBD. (B) LysSA11-CBD binds to *S. aureus* ATCC 13301 cells. EGFP-LysSA11-CBD, treatment of *S. aureus* ATCC 13301 with EGFP-LysSA11-CBD; EGFP only, treatment of *S. aureus* with EGFP alone. Bright field (left), fluorescence (middle), and merged (right) images are shown.
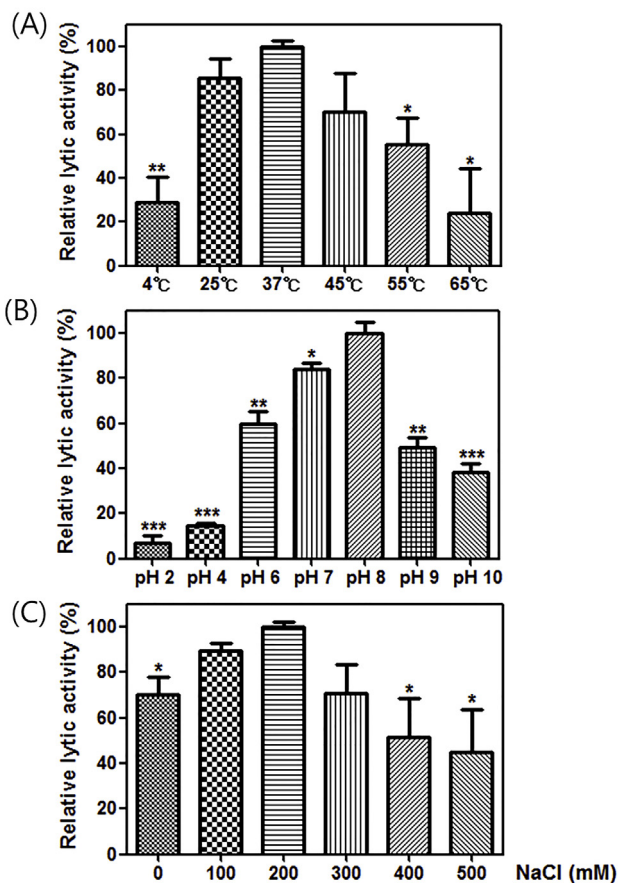
Fig. 4. Effects of pH, temperature, and NaCl on the lytic activity of LysSA11. Effects of (A) temperature, (B) pH, and (C) NaCl concentration on the lytic activity of LysSA11 against *S. aureus* ATCC 13301 cells. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. The asterisks indicate significant differences (***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$).

lytic activity of LysSA11 was determined in the presence or absence of different metal ions. Lytic activity was decreased for EDTA-treated LysSA11, suggesting that LysSA11 requires metal ions for full activity. However, when 1 mM Ca^{2+} was added to the EDTA-treated endolysin, the activity was approximately 150% of that of the control (Fig. 5). Intriguingly, the activities of LysSA11 treated with other metal ions (i.e., magnesium, manganese, copper, and zinc) were similar to that of the EDTA-treated sample, indicating that the dependence of LysSA11 on divalent metal ions is calcium-specific. In accordance with this finding, several CHAP domain-containing staphylococcal endolysins (Donovan et al., 2006; Fenton et al., 2011; Gu et al., 2014) have been shown to require calcium ions for their activity.

To determine the spectrum of LysSA11 lytic activity, 25 different bacterial strains including MRSA and several other representative Gram-positive and Gram-negative bacteria were tested by the lytic assay (Table 1). Although no lytic activity was observed against any other strain, lytic activity was exhibited against all staphylococcal strains tested, including MRSA. Thus, LysSA11 has an activity spectrum specific to staphylococcal species, in accordance with the binding spectrum of the LysSA11-CBD.

3.4. Antibacterial ability of LysSA11 against S. aureus in milk and ham

We next evaluated the suitability of LysSA11 as a biocontrol agent for *S. aureus* in food. Antibacterial activity against a MRSA (*S. aureus* CCARM 3089) strain in pasteurized milk or ham was examined at various endolysin concentrations (1.125–9 μM). Milk and ham are known to have a high possibility of *S. aureus* contamination (Hennekinne et al., 2012; Richards et al., 1993). Furthermore, these foodstuffs are appropriate models for determining LysSA11 activity because they are neutral/moderately acidic (milk; pH 6–7) or sodium-containing (ham) (Fig. 4B and C). The number of artificially inoculated MRSA cells was not reduced in the negative control group (untreated), but was reduced significantly by LysSA11 treatment (*see below*). No viable resistant cells were recovered one day after endolysin treatment, confirmed by direct plating onto a BPA plate.

In milk stored at refrigeration temperature (4 °C), significant
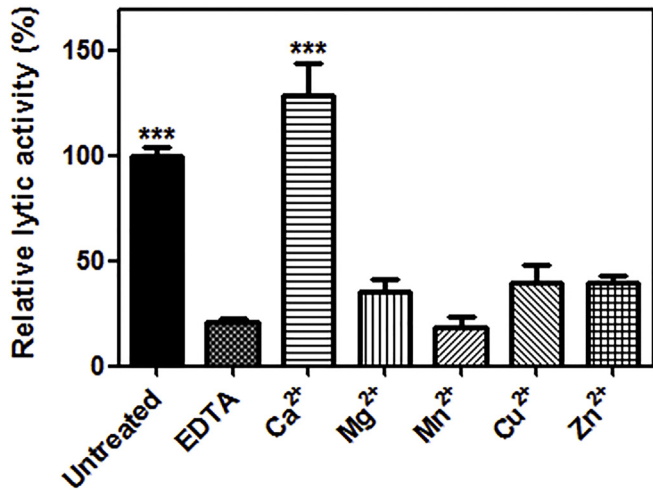
Fig. 5. Effects of metal ions on the lytic activity of LysSA11. Relative lytic activities were calculated by comparing the activity of each ion-treated group to that of non-treated group. LysSA11 was pre-treated with EDTA to chelate any adsorbed ions. Values represent means and standard deviations of triplicate assays; asterisks indicate significant differences (***, $P < 0.0001$).

inhibitory effects ($P < 0.05$) were shown within 15 min by 3.375 μM of LysSA11. Moreover, viable cells were reduced to undetectable levels at 1 h by treatment with 9 μM of LysSA11 (Fig. 6A). At room temperature (25 °C), significant inhibitory effects ($P < 0.05$) were shown within 15 min of treatment with 3.375 μM of LysSA11, similar to the findings at 4 °C. However, the viable cells were more rapidly reduced to undetectable levels (within 30 min) by treatment with 9 μM of LysSA11 (Fig. 6B). These results suggest that LysSA11 in milk works more efficiently at 25 °C than at 4 °C, in

accordance with the result shown in Fig. 4A that LysSA11 showed higher activity at 25 °C than at 4 °C.

When ham stored at 4 °C was treated with 3.375 μM LysSA11, viable staphylococcal cells were reduced to undetectable levels within 30 min of treatment. Moreover, treatment with 1.125 μM LysSA11 resulted in significant killing of bacteria ($P < 0.0001$) within 15 min (Fig. 6C). At 25 °C, 1.125 μM of LysSA11 was sufficient to reduce the number of staphylococcal cells to undetectable levels within 30 min (Fig. 6D). These results suggest that LysSA11 in ham works more efficiently at room temperature than at 4 °C. LysSA11 also worked more efficiently in ham than in milk, probably due to the difference in endolysin treatment methods. Specifically, LysSA11 was directly applied to the target cells on the surface of the ham, while the endolysin was diluted when added to the milk. The results suggest that LysSA11 would work better if it is concentrated on the contaminated surface of solid foods rather than used in liquids. A few studies have studied the application of staphylococcal endolysins to milk (Garcia et al., 2010; Obeso et al., 2008) and found that treatment with 160 U/mL (88 μg/mL) of LysH5 endolysin for 1 h resulted in an approximately 1-log reduction of *S. aureus* cells per milliliter (Obeso et al., 2008). In the present study, LysSA11 treatment (3.375 μM, 1 h) reduced the number of staphylococcal cells in milk by about 2.53-log per milliliter. LysSA11 also showed strong activity in ham artificially contaminated with staphylococcal cells. These data suggest that LysSA11 is a promising biocontrol agent as a food antimicrobial to control *S. aureus* in dairy or ham products.

3.5. Disinfection efficacy of LysSA11 against S. aureus on cooking utensils

Cross-contamination during food processing or preparation is a major cause of foodborne illness outbreaks. In particularly, insufficient cleaning of cooking utensils is the most common fault
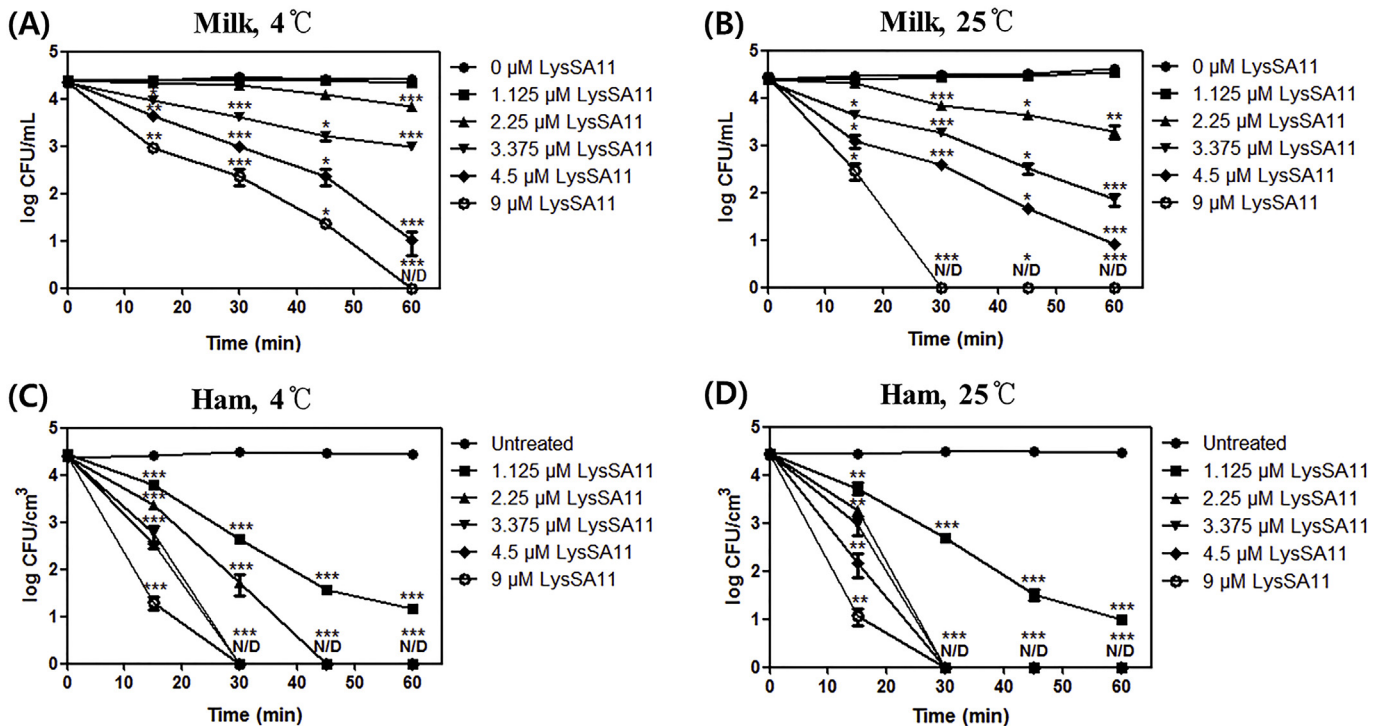

Fig. 6. LysSA11-mediated elimination of methicillin-resistant *S. aureus* CCARM 3089 at (A, C) 4 °C and (B, D) 25 °C in (A, B) pasteurized whole milk and (C, D) ham. The numbers of *S. aureus* CCARM 3089 cells without LysSA11 treatment (negative control) and with LysSA11 1.125 μM, 2.25 μM, 3.375 μM, 4.5 μM, and 9 μM treatment were counted. Values are the means of three independent experiments with standard deviations. The asterisks indicate significant differences (***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$). N/D, not detected.

reported (Bennett et al., 2013; Kadariya et al., 2014). When two different kinds of cooking utensils artificially contaminated with MRSA were treated with LysSA11, the amount of bacteria killed was proportional to the amount of endolysin added. Of particular note, the endolysin effects were not significantly different on the polypropylene plastic cutting board versus the stainless steel knife (Fig. 7). Treatment of the surface of the polypropylene plastic cutting board or stainless steel knife with 1.35 μM LysSA11 resulted in complete elimination of the contaminating MRSA within 30 min, indicating that utensil material does not significantly impact the effectiveness of LysSA11 against staphylococcal cells. Staphylococcal endolysins have not yet been used as a disinfectant; this is the first successful application of a staphylococcal endolysin to the surface of cooking utensils. Thus, we propose that the LysSA11 endolysin is an ideal decontaminant for removing *S. aureus* from the surfaces of cooking utensils, especially those made of plastic or stainless steel.

4. Conclusions

Our data revealed specific binding activity and lysis activity of the novel CBD-containing endolysin LysSA11 against the notorious foodborne pathogen, *S. aureus*. LysSA11 retained about 30% of its activity under refrigerator temperature and 50% of its activity between pH 6 and 9, suggesting the usability of LysSA11 in diverse foods stored at a refrigerator temperature. Indeed, LysSA11 significantly reduced the number of MRSA from milk and ham at 4 °C. Further studies about the combination of LysSA11 with other antimicrobial agents might be able to elevate the effectiveness of the endolysin. Moreover, utensils made of polypropylene plastic or stainless steel were also protected from the MRSA contamination by LysSA11 treatments. Because the activity of LysSA11 was stably maintained at the room temperature, cross-contamination of *S.*

aureus during the food processing or cooking could be effectively prevented when the utensils and equipment were prophylactically treated with the LysSA11. In conclusion, the staphylococcal-specific endolysin LysSA11 could be employed as both antimicrobials and practical sanitizers for food safety.

Acknowledgements

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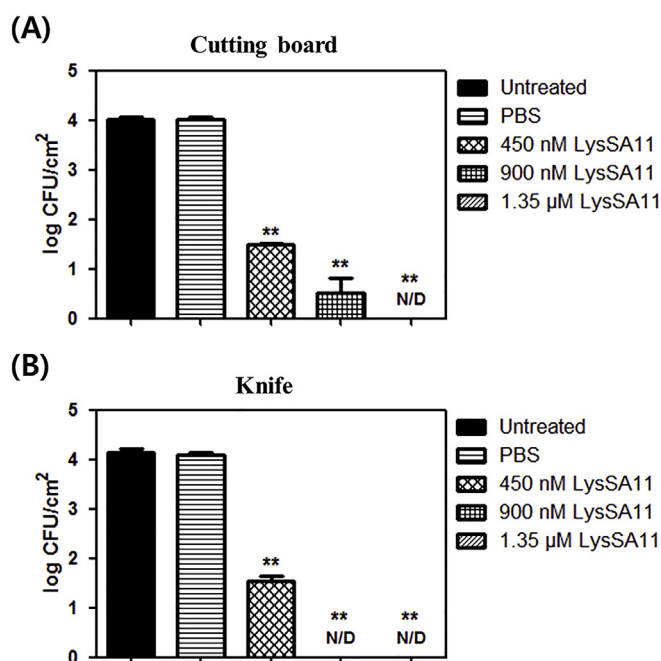
Fig. 7. Antibacterial activity of LysSA11 endolysin against methicillin-resistant *S. aureus* CCARM 3089 on a (A) polypropylene plastic cutting board and (B) stainless steel knife. The numbers of *S. aureus* CCARM 3089 cells without LysSA11 treatment (negative control), with PBS, and with LysSA11 450 nM, 900 nM, and 1.35 μM treatment were counted. Each column represents the mean and standard deviation of triplicate assays; asterisks indicate significant differences (**, $P < 0.01$). N/D, not detected.

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